ISOLATION AND PROPERTIES OF TOBACCO MOSAIC AND OTHER VIRUS PROTEINS

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IN ancient times disease was regarded as being due to supernatural agencies such as evil spirits and the will of the gods. Later the idea that disease resulted from natural causes such as comets, earthquakes, and the change of the seasons was quite widely held. About 100 B. C. Varro[1] and Columella[2] each expressed the idea that certain diseases might be caused by invisible living agents, but there was no experimental proof and the idea was not accepted. The writings of Fracastorius[3] about 1500 containing his theory of contagion, accurate descriptions of plague and of rabies, and a notation of the immunity that follows an attack of smallpox or of measles constituted an important advance, despite the fact that he made no special reference as to whether the contagious agents were living or non-living. It was not until about 1680 when, through the wonderful work of Leeuwenhoek,[4] the world of microscopic living organisms really became known. Although he described bacteria in 1683, it was over fifty years before his work was generally regarded as confirmed. Many workers considered these micro-organisms to be capable of causing disease, yet about a hundred years elapsed before their connection with disease was proved experimentally.

During the latter half of the nineteenth century there arose great controversies over the germ theory of disease, the nature of fermentation, and the age-old question of spontaneous generation, the latter of which had survived the blows administered by Redi[5] in 1668 and by Spallanzani[6] in 1776. These diverse yet related controversies were resolved through the brilliant researches of Pasteur,[7] Koch,[8] Tyndall,[9] Davaine[10] and others. It was proved for the first time that disease could be caused by a small living organism. The germ theory of disease emerged so triumphantly and was accepted so completely that thereafter there was a

definite tendency to regard all infectious diseases as being caused by bacteria. Thus, in 1892, when Iwanowski[11] discovered that the juice of a plant diseased with tobacco mosaic remained infectious after being passed through a filter which removed all of the known living organisms, he did not regard it as being especially significant and concluded that the disease was bacterial in nature. Six years later Beijerinck,[12] in a well planned and executed research, repeated and confirmed Iwanowski's experiments, and in addition demonstrated by serial passage of the filtrate that the disease was not due to a bacterial toxin. Beijerinck realized the significance of his results and referred to the infectious agent, not as being bacterial in nature, but as a contagious living fluid. Although he wished to differentiate it from ordinary bacteria, he too was thoroughly imbued with the idea that the infectious entity was living. These filtration experiments are regarded as the first demonstration of an agent that is now known as a virus. The same year Loeffler and Frosch[13] announced that the infectious agent of the foot-and-mouth disease of cattle would also pass through filters capable of retaining bacteria, and in 1901 similar results were obtained with the agent causing yellow fever in man.[14]

Characteristic Properties of Viruses

Since 1901 hundreds of the diseases of man, animals, and plants have been found to be caused by viruses. Included in this group are such diverse diseases as smallpox, rabies, psittacosis, fever blisters, epidemic encephalitis, yellow fever, poliomyelitis, fowl pox, hog cholera, dog distemper, equine encephalitis, certain types of tumorous growths in fowls and other animals, various yellows and mosaic diseases of plants, and possibly the transmissible lysis of bacteria. The earliest recognized property of the agents causing these diseases that was used to differentiate them from bacteria, namely, their filterability, has long since been generally recognized as untenable, for some of these viruses will not pass filters which will permit known organisms to pass. However, it has been replaced by certain other properties that are regarded today as characteristic of viruses. These emphasize the intimate relationship that exists between viruses and host cells, the fact that many virus-infected cells contain inclusion bodies, the fact that no virus has been grown on cell-free media, the fact that most but not all virus diseases are followed by a lasting immunity in recovered hosts, and the fact

that as a group viruses are smaller than ordinary bacteria. It should be emphasized that no single one of these properties may be used to differentiate viruses from bacteria and that, despite the attempted separation based on the properties just mentioned, viruses have nevertheless been generally considered as merely small ordinary living organisms, somewhat similar to the bacteria.

The fact that viruses may multiply or reproduce, that they may change or mutate and adapt themselves to new conditions, that they are specific in their action in that a given virus occurs or causes disease only in certain hosts, and that a lasting immunity follows most virus diseases has been used in arguments for the living nature of viruses, for these properties have been generally regarded as characteristic of living things. There were but few dissenters, and the large majority of the workers in the virus field saw no reason why viruses should not be considered small invisible living organisms. This conviction became even stronger with the discovery that some viruses were actually larger than certain bacteria.[15] However, in 1931 Galloway and Elford[16] reported that the virus of the foot-and-mouth disease of cattle was only about 8-12 mμ in diameter, only slightly larger than the familiar hemoglobin molecule and actually several times smaller than some of the hemocyanin protein molecules. Here, therefore, was a living organism that was smaller than a protein molecule! Evidence of a growing unrest and general dissatisfaction with this situation became noticeable in the writings of the time. Some of the virus workers realized the dilemma that had presented itself and attempted to find a solution. Thus, Burnet and Andrewes[17] in 1933 suggested that viruses might be divided into two groups, one consisting of organized living agents and the other of unorganized, supposedly non-living materials. Then they concluded that viruses affecting animals, presumably including the troublesome foot-and-mouth disease virus of molecular dimensions, were living organisms and belonged in the first group, whereas the bacteriophages ranging in size from about 10 mμ to 100 mμ and the large fowl tumor virus were placed in the second group. They apparently created the second group especially for the unusually small viruses and then neglected to use it for the foot-and-mouth disease virus. Rivers[18] was also troubled by the small size of this virus, and in his Harvey Lecture delivered four years ago suggested a division of viruses according to size. He considered that some might be minute living organisms, others representatives of a form of life unfamiliar to us, and still others non-living agents.

Selection of Tobacco Mosaic Virus

We thus have the unusual situation in which viruses, originally grouped together because of characteristic and similar properties, are subdivided solely on the basis of size, merely because it is repugnant to consider agents the size of protein molecules as living. A perusal of Figure 1, which shows the comparative sizes of entities ranging from the red blood cell, through bacteria and viruses, down to the egg albumin molecule, immediately reveals certain inherent difficulties in attempting such a subdivision. It may be seen that viruses form an unbroken series with respect to size from living organisms to protein molecules, and at either end there is an overlapping. Certain viruses are larger than accepted living organisms and other viruses are smaller than protein molecules. Where shall the lines subdividing the viruses be drawn?

I do not feel that we should permit ourselves to be drawn too far afield simply that we may preserve in our minds the supposed sanctity of the division between the living and the non-living. Let us, if necessary, revise our ideas and cease attempting to meet new situations with old definitions. So far as we know at the present time, viruses are similar in nature and there is no justification for attempting to subdivide them solely because of size. I consider, therefore, that the discussion this evening which is to center about tobacco mosaic virus is pertinent not only to this virus but to other viruses as well. Despite the fact that it affects only plants and is among the most stable of all viruses, it may be considered a representative virus with respect to the characteristic virus properties, and there is no reason to believe that knowledge gained through a study of tobacco mosaic virus may not, within certain limits, be applied to other viruses.

The unusual stability of tobacco mosaic virus has caused it to be an excellent subject for experimentation and as a consequence it has been used in numerous researches. One of the most extensive of the earlier studies was that of Allard[19] who, during the years 1916 to 1918, determined the effect of many different reagents on virus activity in an effort to learn something of the nature of the virus. In 1927 Vinson[20] undertook the purification of tobacco mosaic virus and with Petre reported in 1929 and 1931 on various procedures useful in separating the virus from much extraneous material. The crystalline material which was mentioned by Vinson and Petre in 1931 and which has been referred to editorially as

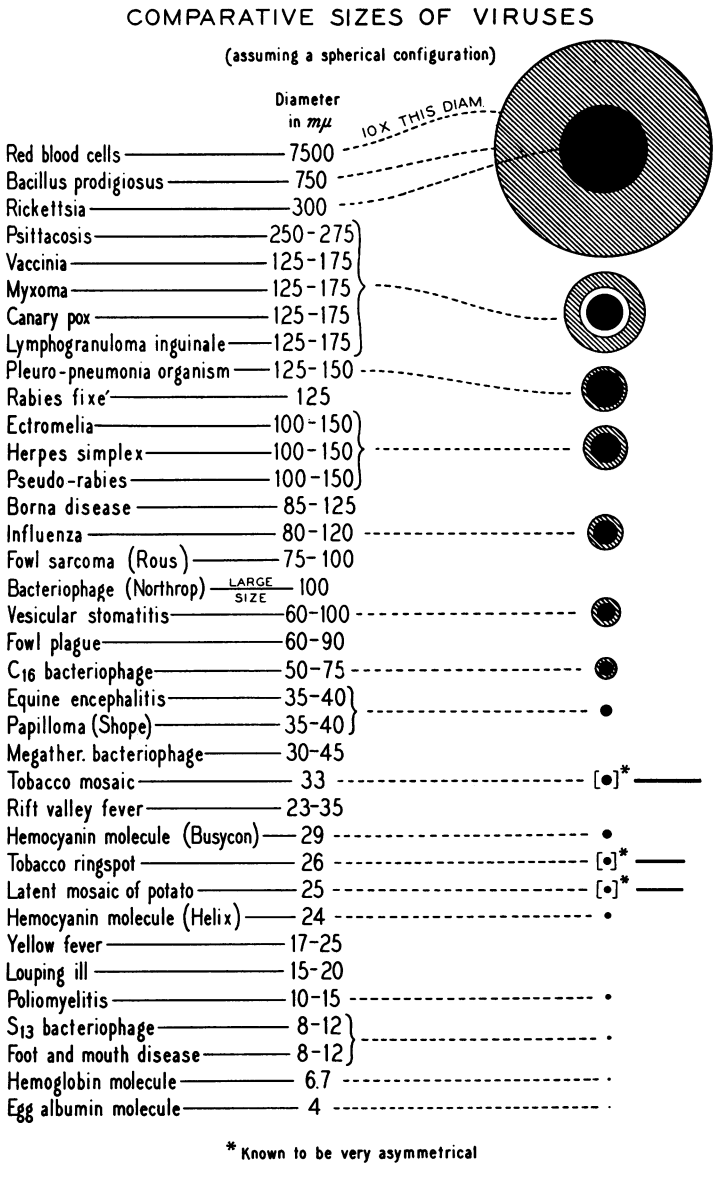

Fig. 1—A chart showing the relative sizes of several selected viruses including bacteriophages, as compared to those of the red blood cell, *Bacillus prodigiosus,* rickettsia, pleuropneumonia organism, and protein molecules. Three of the viruses have markedly asymmetrical configurations and are represented by the broad lines drawn to the right of the circles. Although these lines are drawn to represent rods having a circular cross section and a volume equivalent to that of the sphere, they should be regarded merely as illustrating markedly asymmetric particles. The figures used in the chart have been arbitrarily selected from the data of Elford, McIntosh, Bauer, Schlesinger, Svedberg, and others.

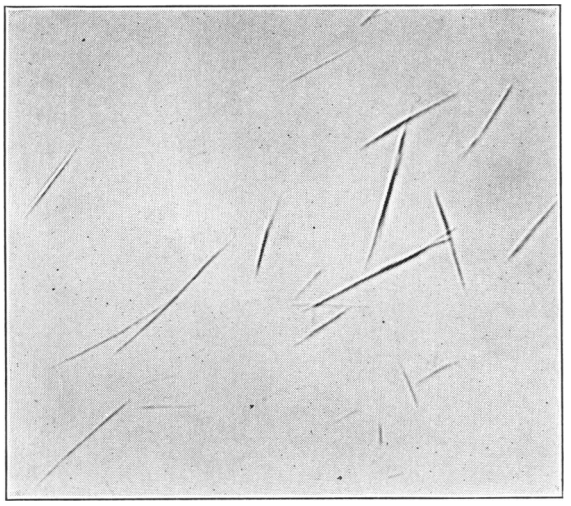

Fig. 2—Crystalline tobacco mosaic virus protein prepared by Dr. H. S. Loring. x 675 (Photo., J. A. Carlile).

crystalline virus actually consisted largely of inorganic matter having no connection with the virus. Vinson and Petre found the crystals to contain about 33 per cent ash and to lose activity on recrystallization, and they concluded that the crystals did not represent pure virus. The active crystalline material containing no demonstrable nitrogen, which was obtained by Barton-Wright and McBain[21] by means of Vinson and Petre's lead acetate method, was found by Caldwell[22] to consist of virus adsorbed on crystals of inorganic material.

Isolation and Properties of Tobacco Mosaic Virus Protein

Early in 1935 there was isolated for the first time an unusual, high molecular weight crystalline protein which possessed the properties of tobacco mosaic virus and which since has become known as tobacco mosaic virus protein.[23] The crystals of this protein are reproduced in Figure 2. This material was isolated by means of a chemical procedure that involved the use of one step of Vinson and Petre's lead acetate method but which was based chiefly on the general methods of protein chemistry that had been used so successfully by Northrop[24] and associates for the isolation of enzymes. The two properties of this protein that immediately set it apart from other proteins were that it carried high virus activity and that it had a molecular weight greater than that of any other known protein. One cc. of a solution containing only one part of

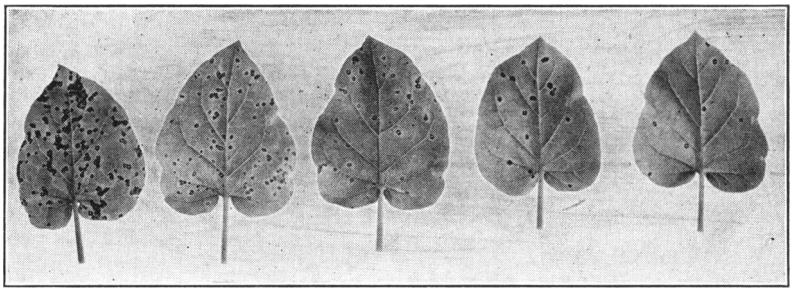

Fig. 3—Local lesions on leaves of plants of *Nicotiana glutinosa* showing effect of diluting juice containing tobacco mosaic virus (1:1, 1:3, 1:10, 1:100, and 1:1000). (From Holmes.[25])

this virus protein in ten billion parts of phosphate buffer was usually found infectious. The disease produced in plants by this as well as by more concentrated solutions was the typical tobacco mosaic disease, and from such plants more virus protein of the same kind as that introduced could be isolated. The activity of the virus protein may be determined with considerable accuracy by means of the Holmes[25] local lesion method. About forty-eight hours after a virus preparation is rubbed, by means of a bandage gauze pad, over the upper surfaces of the leaves of certain plants, necrotic lesions or spots appear. As may be seen in Figure 3, the number of lesions or spots may be used as an index of the amount of virus in the inoculum, for the more virus there is present the greater is the number of lesions that is obtained. When the method is suitably standardized by means of inoculating an unknown and control preparation on different portions of the same set of leaves, the virus activity may be determined with an error no greater than about 10 per cent.[26] It may be noted that Calmette and Guérin[27] in 1901 used the local lesion response as a measure of the potency of preparations of vaccinia virus.

Preliminary experiments on the diffusion and osmotic pressure of the virus protein indicated that it had a molecular weight of the order of several millions. Since these methods are not well suited for such huge molecules, a sample was sent to Dr. Svedberg for an ultracentrifugal analysis. The molecular weight based on a dissymmetry constant of 1.3 was found to be seventeen millions.[28]* The question that was immediately asked and that became of paramount importance was, "Is this

* Recent data indicates a dissymmetry constant of 2.52 which would give a molecular weight of about fifty millions. See: Lauffer, M. A. The molecular weight and shape of tobacco mosaic virus protein, *Science*, 1938, *84*:469.

unusual, high molecular weight protein tobacco mosaic virus?" On the present occasion I think that we may seek the answer to this question by means of the application of a chemical version of Koch's postulates. You are all familiar with the methods that are used by the bacteriologist to isolate and recognize a given organism. You are probably less familiar with the methods of the chemist, yet I can assure you that, because the compounds with which the chemist works have certain definite characteristic properties, they can be recognized with an accuracy that is no less than that involved in the bacteriologist's recognition of an organism. Tobacco mosaic virus protein has a definite and specific virus activity, chemical composition, x-ray diffraction pattern, ultraviolet light absorption spectrum, isoelectric point, sedimentation constant, diffusion constant, solubility, gives the usual protein color reactions, and is precipitated by the usual protein-precipitating agents.[23] In solution it has a characteristic opalescence, shows a marked Tyndall cone, exhibits strong double refraction of flow, and takes on a characteristic satin-like sheen when stirred. Concentrated solutions on standing form two definite layers that possess different physical properties. The protein has characteristic heat and pH stability ranges and is denatured only under certain definite conditions. Solutions containing but 10^{-7} gm. of the protein give a specific precipitin reaction with antiserum to the protein. These are some of the properties that are used to characterize the protein. So far as is known, this set of properties is not possessed by any other entity. The entire science of chemistry is built upon the recognition of substances by means of such properties, and I consider the recognition of tobacco mosaic virus protein by means of its properties to be as valid as the means used by the bacteriologist to identify a given organism.

Since there is no difficulty in recognizing the virus protein, we may proceed with our consideration from the standpoint of Koch's postulates. In the first place, this same protein possessing the same set of characteristic properties should be present in every case of the tobacco mosaic disease. During the past three years several hundred batches of mosaic-diseased Turkish tobacco plants were examined, and protein possessing identical physical, chemical, biological, and serological properties was obtained from all batches of plants worked up under comparable conditions. When different methods of purification were used, the protein was found to differ slightly depending upon the method used for isolation. However, in the case of this first postulate an even more severe

test may be applied, for tobacco mosaic virus has one of the widest host ranges known. It causes disease not only in several species of tobacco but also in plants such as spinach and phlox which are so distantly related that their normal constituents give no cross precipitin reaction with antiserum to the normal constituents of tobacco. Now, what do we find when we examine mosaic-diseased plants belonging to different species? The protein isolated from Burley tobacco, tomato, common nightshade, petunia, spinach, and phlox plants diseased with tobacco mosaic has been found to possess, so far as determined, the same physical, chemical, biological, and serological properties as those of the tobacco mosaic virus protein first isolated from diseased Turkish tobacco plants. The first postulate is fulfilled quite satisfactorily, therefore, since the virus protein has been found in every case of the tobacco mosaic disease.

Viruses have never been grown in the absence of cells, hence it is impossible to fulfill the second postulate as stated. However the third and fourth postulates are fulfilled quite readily, for I have already mentioned the fact that inoculation of any susceptible host with the virus protein results in the production of the typical tobacco mosaic disease and from these plants may then be isolated more of the same kind of protein as that used for inoculum. The successful application of Koch's postulates to tobacco mosaic virus depends, therefore, only upon whether or not means for satisfying the essence of the requirements of the second postulate can be found. The original purpose of this postulate was the demonstration, beyond a reasonable doubt, that the infectious agent could be obtained in pure form. It so happens that the question of the purity of the virus protein can be attacked best, not by cultivation methods, but by physical, chemical, and serological methods. However, if a material can be proved pure by these methods, I think that there is valid reason for considering the second postulate to have been fulfilled. From a chemist's standpoint, the question of purity is of the utmost importance, for if the infectious material is pure it follows directly that it is the virus. This question has, therefore, been studied at considerable length by means of as many different types of procedures as it has been possible to devise. The isolation of a protein having the same physical, chemical, biological, and serological properties from many different batches of diseased Turkish tobacco plants and from many other species of diseased plants is, of course, good presumptive evidence that a single substance is under consideration. There are, however, several more direct methods for deter-

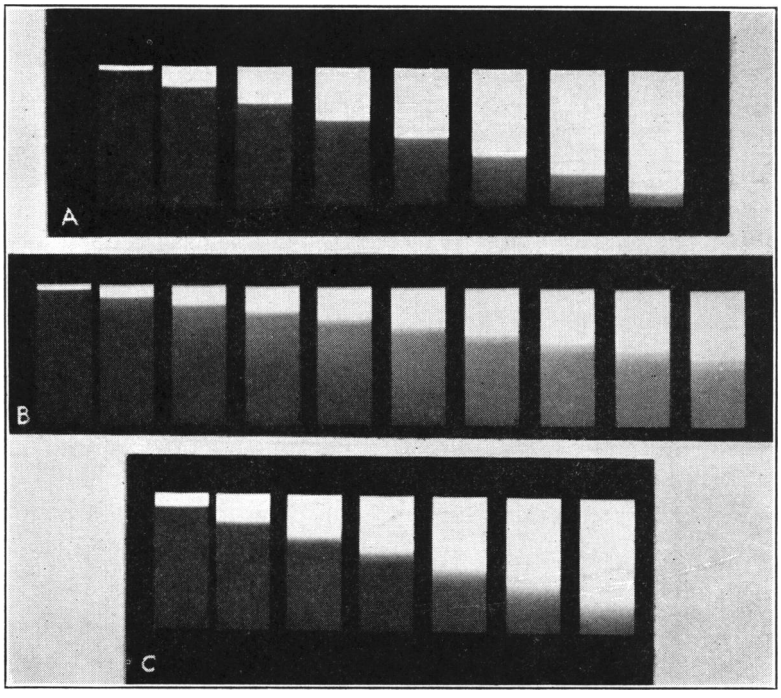

Fig. 4—Sedimentation pictures prepared by Dr. Wyckoff of solutions of tobacco mosaic virus protein. (a) Protein isolated by mild means such as by differential centrifugation, (b) following development of a second component caused by allowing protein to stand in the presence of salt, and (c) following more extensive treatment with salt.

mining the homogeneity of a preparation. Repeated crystallization with retention of constant properties has long been considered a criterion of purity. Despite the fact that this procedure has occasionally proved none too efficacious in the case of certain proteins, it seemed desirable to apply it to the virus protein. It was found that the virus activity of protein crystallized once was unchanged following fifteen successive crystallizations or following drastic fractional crystallization, provided the experiments were carried out rapidly in the cold and with low concentrations of salt. Although these experiments may not be considered conclusive proof that the virus protein is pure, they do demonstrate that it is impossible to detect an impurity by this method of fractionation. Eriksson-Quensel and Svedberg[28] found the protein to be completely homogeneous with respect to its electrochemical behavior. These workers

also made an ultracentrifugal analysis and found some of the earlier preparations to be somewhat inhomogeneous with respect to sedimentation constant. Wyckoff,[29] using some of our later preparations that were prepared by a less drastic method, found the protein to give the single sharp boundary shown in Figure 4a that is characteristic of a single molecular species. The protein forms a second component on standing in the presence of a little salt (Figure 4b), and on more extensive treatment becomes quite inhomogeneous (Figure 4c). The remarkable homogeneity of carefully prepared tobacco mosaic virus protein with respect to sedimentation constant and electrochemical behavior provides additional evidence for the purity of the protein.

The ultraviolet absorption spectrum of the virus protein was determined by Dr. Lavin[30] and found to have a maximum at about 2600-2650 A°. The absorption spectrum of the virus protein was found to agree essentially with the destruction spectrum of virus activity,[31] that is, just those wave lengths of ultraviolet light that were preferentially absorbed by the protein were exactly the same ones that caused inactivation. There is no doubt but that the light is absorbed by the protein, and the fact that this absorption of energy by the protein results in loss of virus activity is good evidence that the activity is a property of the protein. Further evidence relating activity and protein was obtained by denaturation of the virus protein by different methods. If the activity is a specific property of the protein, partial or complete destruction of the protein should result in a corresponding loss of virus activity. It was found that partial or complete denaturation of the protein by heating or by the use of acid, alkali, or chemical reagents was always accompanied by a corresponding loss of virus activity. For example, as may be seen in Figure 5, the sedimentation constant and virus activity of the protein remain unchanged following adjustment of solutions to hydrogen ion concentrations between about pH 2 and pH 8.[32,33] At more acid or alkaline reactions the virus activity is lost rapidly, and at exactly the same hydrogen ion concentration the protein is denatured and broken up into material having much lower sedimentation constants.

Correlation of Virus Activity with Protein

With the present method of inoculation, solutions containing from about one hundred to about one million molecules of the protein per cc. are required to cause infection. It may be argued, therefore, that the high

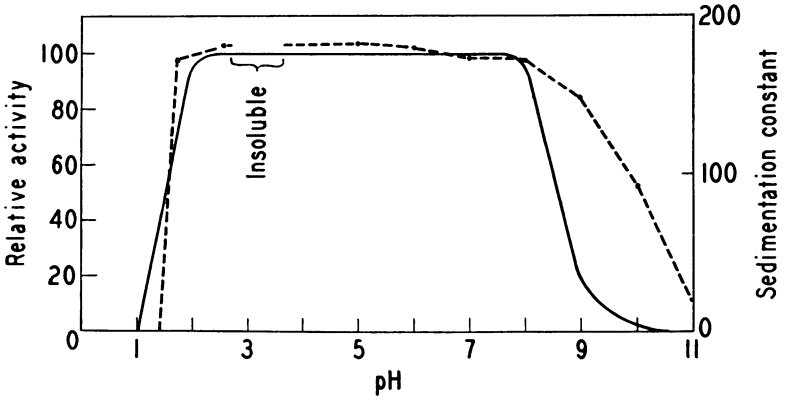

Fig. 5—pH stability range of tobacco mosaic virus protein as measured by virus activity (solid line) and by sedimentation constant (dotted line). (Drawn from data of Best and Samuel,[32] Stanley,[23] Eriksson-Quensel and Svedberg[28] and Wyckoff.[33])

molecular weight protein is not the virus and that the activity is due to virus adsorbed on the protein. It is unlikely that it will be possible to demonstrate experimentally whether or not one molecule of high molecular weight protein may cause infection until the method of inoculation is improved. However, there are other ways of approaching this question experimentally. The high molecular weight protein is isoelectric at about pH 3.5 and possesses a negative charge at more alkaline reactions and a positive charge at more acid reactions. It can be shown that, whether a hypothetical virus-carrying entity possess a constant negative charge, a constant positive charge, or be isoelectric at some hydrogen ion concentration other than that of the isoelectric point of the protein, it will possess the same charge as the high molecular weight protein at some reaction more acid or alkaline than pH 3.5. At the hydrogen ion concentration where the high molecular weight protein and the hypothetical virus entity possess the same charge, they should be separated from one another. Then, if the hypothetical entity is considerably larger or smaller than the high molecular weight virus protein, it should be possible to effect physical separation of the two by centrifugation. The virus protein is insoluble at its isoelectric point and may be readily sedimented to give a supernatant liquid that contains no protein and possesses no virus activity. Furthermore, as may be seen from Table 1, it was found experimentally that, when solutions of virus protein were centrifuged at pH 2.4 where the protein possesses a positive charge and at pH 6.7

where it possesses a negative charge, so that about 85 to 95 per cent of the protein was removed from the upper portions of the supernatant liquids, the virus activity of the separated upper and lower portions of the solutions was proportional to the amount of high molecular weight protein that they contained. These results prove that, when negatively charged protein ions, positively charged protein ions, or neutral protein are subjected to centrifugation, the virus and the protein sediment at exactly the same rate. These experiments provide, therefore, a very strong argument against the hypothesis that the virus activity is due to a separate entity adsorbed on the high molecular weight protein or,

TABLE I

Correlation of virus activity and protein on centrifugation of tobacco mosaic virus protein at pH 2.4 and 6.7[a].

pH during centrifugation	*Test No.*	*Protein concentration after centrifugation (mg. per cc.)*	*Portion of centrifuged sample used*	*Protein concentration used for tests (gm. per cc.)*	
				10^{-4}	10^{-5}
2.4	1	1.2	Upper portion	57.9[b]	25.3
		28.3	Lower portion	62.1	30.4
			No. of half leaves	52	52
			M.D./S.D.[c]	0.96	2.34
	2	1.2	Upper portion	58.1	30.6
		28.3	Lower portion	68.3	30.9
			No. of half leaves	56	56
			M.D./S.D.	2.51	0.15
6.7	1	1.2	Upper portion	145.0	74.8
		16.8	Lower portion	161.2	82.8
			No. of half leaves	56	56
			M.D./S.D.	2.02	1.89
	2	1.2	Upper portion	70.3	22.0
		16.8	Lower portion	79.9	25.8
			No. of half leaves	52	52
			M.D./S.D.	2.02	2.03

[a]Tests following dilution of lower portions to same protein concentration as in the corresponding upper portions of centrifuged samples. All dilutions were made with 0.1 M phosphate buffer at pH 7. *Phaseolus vulgaris* was used as the test plant.

[b]Numbers opposite a given preparation represent the average number of lesions per half leaf obtained on inoculation with the designated preparation and concentration. A given preparation was administered to the right halves of half of the leaves and to the left halves of the remaining leaves in each test.

[c]To show a significant difference between the mean number of lesions in any one experiment, the ratio of the mean difference (M.D.) to the standard error of the mean difference (S.D.) should not be less than 2.1.

for that matter, to a dissociable active group attached to the protein, and are direct evidence that the virus activity is a specific property of the protein.

Another method of approaching the question as to whether or not virus is merely adsorbed is to mix various proteins with the virus protein and determine whether or not the virus protein can be recovered with its characteristic virus activity. If virus were merely adsorbed on the high molecular weight protein, it seems likely that some of it would be lost and remain with the other proteins. However, it was found that from mixtures of virus protein with egg albumin, globin, trypsin, or pepsin it was always possible to recover the protein with its virus activity unchanged. Gratia and Manil,[34] working with mixtures of virus protein and phage protein, were also able to demonstrate that the two could be separated by centrifugation or by crystallization of the virus protein. They found, for example, that following four crystallizations of the virus protein the phage titer had dropped to 1/100 its original value. Basset, Gratia, and co-workers[35] also studied the effect of high pressure on virus activity, the ability to precipitate with antiserum, and the ability to crystallize, of purified virus protein. They found that these properties were unaffected by pressures up to about 6,000 atmospheres, but that at 8,000 atmospheres' pressure each of these properties was practically destroyed.

Double Refraction of Flow and Layering Phenomenon

There is another interesting and unusual property of the virus protein that may well be considered here, because it also results in a fractionation of the protein. Takahashi and Rawlins[36] noted in 1932 that the juice of mosaic-diseased plants was doubly refracting when made to flow, whereas the juice of normal plants failed to exhibit this phenomenon. Recently these workers also found that the suspensions and solutions of crystalline tobacco mosaic virus protein show double refraction of flow.[37] Evidence has been obtained by Bawden, Pirie, and co-workers[38] and in the writer's laboratory by Dr. Lauffer[39] that the molecules of virus protein are markedly asymmetric and have a length between ten and thirty times greater than their cross section. The rod-like shape of the virus protein molecules is apparently responsible for the separation of rather concentrated solutions of virus protein into two distinct layers, a phenomenon that was first reported by Bawden and Pirie.[38] As may be

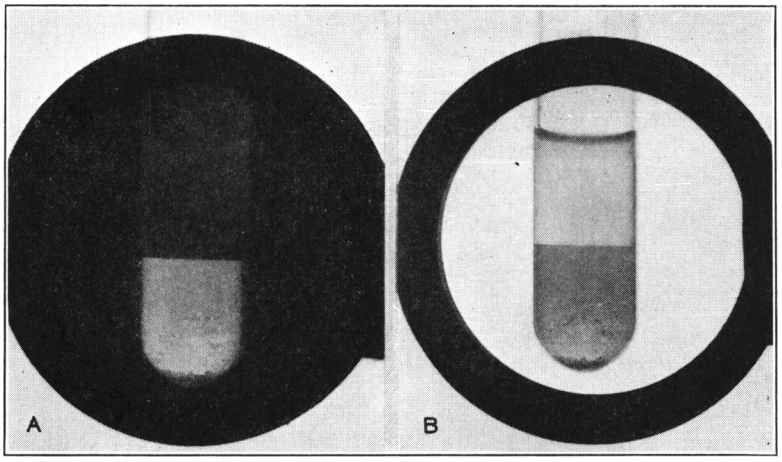

Fig. 6—Photograph in polarized light of a concentrated solution of tobacco mosaic virus protein that has been allowed to stand. (a)Test tube with crossed Polaroid plates on opposite sides. (b)Test tube with parallel Polaroid plates on opposite sides. The lower layer is spontaneously doubly refracting, whereas the upper layer is not. (Photograph by J. A. Carlile.)

seen from Figure 6, the line of demarcation between the layers is quite sharp. The upper layer shows double refraction only when made to flow and has a lower protein concentration than the lower layer. The latter is spontaneously doubly refracting apparently because, as the rod-shaped virus protein particles become sufficiently concentrated, they lose their ability to rotate about their shorter axes and become orientated. The lower layer appears to consist of a three dimensional mosaic of regions arranged at random to each other but in each of which all of the rod-shaped particles are orientated and are parallel to one another. Under the polarizing microscope the lower layer material when placed on a slide under a cover slip appears to consist of a two dimensional mosaic of doubly refracting areas orientated in different directions. The orientation phenomenon is readily reversible, for lower layer material may be diluted to give upper layer material and the latter may be concentrated to give lower layer material. However, it was of some interest to determine whether or not there was a difference in the virus activity of the two layers. This was done by Bawden and Pirie,[38] and the virus activity of the two layers was found to be exactly proportional to their protein content. Therefore, the fractionation of the protein that may be secured by virtue of this most unusual property also failed to reveal inhomogeneity.

Precipitin and Anaphylaxis Tests

There are still other properties of the virus protein, such as its immunological properties, that may be examined in connection with the question of homogeneity and that should be of especial interest on the present occasion. Purdy (Beale)[40] in 1928 and 1929 noted that the juices of mosaic-diseased plants contain an antigen specific for the virus-containing extracts and not present in the juices of normal plants. Beale,[41] Chester,[42] and other workers extended the serological work and found that antiserum to tobacco mosaic virus not only gave a precipitin reaction with extracts containing this virus and failed to give a precipitin reaction with extracts containing different viruses, but that it also possessed a specific neutralizing effect on tobacco mosaic virus. Despite the fact that no direct proof was available, it was generally considered that the antigen involved in these reactions was the virus itself. It is now known that the antigen was actually tobacco mosaic virus protein. Antiserum to purified virus protein gives a precipitin reaction with solutions containing but 10^{-7} gm. of virus protein per cc. and gives no precipitin reaction with extracts of normal plants or of plants diseased with other viruses. The precipitin reaction is generally regarded as a very sensitive test, and the fact that antiserum to virus protein reacts only with virus protein demonstrates that by this test the protein is homogeneous. It should be noted, however, that it is possible to inactivate the virus protein by very mild treatment such as irradiation with ultraviolet light or reaction with formaldehyde, nitrous acid, or hydrogen peroxide, without seriously altering the serological reactions.[43] Antisera to such inactive proteins give a precipitin reaction with either active or inactive protein and also have a specific neutralizing effect on virus activity. The latter fact is of considerable importance, not only because it may serve as an example of the immunological potentialities in the control of virus diseases, but also because it indicates a close relationship between virus activity and protein.

Another reaction that has been found even more sensitive than the precipitin test is that of anaphylaxis. Chester[44] in 1936, using the Schultz-Dale technique, found that the smooth muscle of the uteri of guinea pigs sensitized to some of the chemically prepared samples of virus protein reacted not only to virus protein but also to proteins extracted from normal plants, but that, following desensitization with proteins

from normal plants, no reaction was obtained with virus protein. These results indicated not only that these samples of virus protein contained a small amount of normal plant proteins as impurity but, more surprising, that the virus protein was not anaphylactogenic. It was found possible to remove the normal plant protein from the virus protein by sedimentation of the latter several times in a high-speed centrifuge or, as was done by Bawden and Pirie,[38] by digestion with trypsin, and to obtain virus protein that gave no cross reaction with normal plant protein. This purified protein was examined by means of the *in vitro* method and found to give no anaphylactic reaction. However, the same sample of virus protein was tested in sensitized guinea pigs *in vivo* by Seastone, Loring, and Chester[45] and found to be anaphylactogenic. These workers concluded that the distribution of the virus protein by the vascular system probably resulted in a more intimate contact with sensitized tissue than was possible in the isolated uterine horn, the exterior of which may be quite impervious to so large a protein molecule. It may be noted, however, that it is possible to prepare virus protein that even by the very sensitive precipitin and anaphylactic tests shows no evidence of containing impurities.

Partial Reactivation of Formolized Tobacco Mosaic Virus Protein

There is another type of experiment that I think should be considered in connection with the question as to whether or not the virus protein is homogeneous. If the protein molecule could be altered chemically with a simultaneous change or loss of virus activity and the molecule subsequently returned to its original structure with a simultaneous return of virus activity, it would serve as strong evidence that the activity is a specific property of the protein. There have been several reports on the reactivation of viruses. Zinsser and Seastone[46] noted in 1930 that occasionally they were able to reactivate, by means of the reducing agent cysteine, preparations of herpes virus that had been inactivated presumably by mild oxidation resulting from exposure to air. Similar results by means of reduction have been reported with this and other viruses by different workers. Vinson and Petre[20] found that safranine or lead subacetate precipitated tobacco mosaic virus in the form of insoluble complexes possessing practically no virus activity, but that by removal of the precipitating agents most of the original activity could be regained. These results have been confirmed by the writer who found in addition

that silver nitrate formed a silver salt of the virus protein that possessed no virus activity, but which could be reactivated by removal of the silver ions by dialysis. However, in all of this work no attempt was made to correlate the structure of the protein with virus activity. It was not even demonstrated that the inactivation caused by safranine, silver, or lead salts was real and not due to insolubility or to toxicity.

Recently, Dr. Ross,[47] working with tobacco mosaic virus protein in the writer's laboratory, completed a research in which not only true inactivation and reactivation of tobacco mosaic virus was demonstrated, but also in which changes in the structure of the protein molecule were followed by chemical means. It was found that the inactivation of virus protein by formaldehyde followed roughly that of a monomolecular reaction and was accompanied by a decrease in amino nitrogen as measured colorimetrically or by means of the Van Slyke gasometric method and by a decrease in the color developed by Folin's tyrosine reagent. The inactivation is not due to the formation of an insoluble or toxic compound, for the inactivated protein was found to be soluble and to be no more toxic than egg albumin or hydrogen peroxide-inactivated virus protein. The inactivation is not due to the presence of free formaldehyde, for the concentration of free formaldehyde was proved to be less than 10^{-5} gm. per cc., a concentration which was found to have no effect on virus activity. When the inactivation reaction was stopped after suitable periods of time by dialysis at pH 7 to remove the excess formaldehyde, it was possible to obtain partially or completely inactivated virus protein that could be reactivated to a marked extent by dialysis at pH 3. The reactivation was accompanied by an increase in amino nitrogen as measured by the color developed with ninhydrin and by an increase in the color developed with Folin's tyrosine reagent. Preparations completely inactive when inoculated at a concentration of one mg. of protein per cc. were found to possess a definitely measurable amount of virus activity following reactivation. Preparations containing 0.1 and 1 per cent, respectively, of the original activity were found following reactivation to contain about 1 and 10 per cent, respectively, of the original virus activity. In other words, it was possible to obtain a 10-fold increase in virus activity by means of the reactivation technique and to demonstrate a simultaneous change in the structure of the protein molecule. Although the exact structural changes have not been determined as yet, it has been possible to measure them, and I feel that it is

highly unlikely that these changes that have been measured are merely fortuitous and have nothing to do with activity. I consider that the reactivation of formolized virus protein brings strong evidence that the virus activity is a specific property of the protein and provides some information relating the structure of the protein to virus activity.

Summary and Discussion of Evidence Relating to Tobacco Mosaic Virus Protein

Now let us consider the available evidence pertinent to the question as to whether or not tobacco mosaic virus protein is pure and hence is the virus. The virus protein isolated from many different batches of diseased Turkish tobacco plants or from other plant species diseased with the mosaic virus was found to possess the same chemical, physical, biological, and immunological properties, and these properties were found to remain unchanged following fractionation of the protein by various procedures. The protein was found to be completely homogeneous with respect to its sedimentation constant and electrochemical behavior. It was found impossible to separate the virus activity from the protein by any one of several procedures. The absorption spectrum of the protein was found to agree essentially with the destruction spectrum of virus activity. It was found impossible to demonstrate the presence of an impurity in purified preparations of virus protein even by the sensitive precipitin and anaphylactic reactions. The pH stability range of the protein was found to coincide exactly with that of the virus activity. Partial or complete denaturation of a protein preparation by any one of several procedures was always found to result in a corresponding loss of virus activity. Finally, it was found possible not only to inactivate and reactivate the virus protein, but also to demonstrate that the inactivation and reactivation were accompanied by simultaneous changes in the structure of the protein molecule. Thus, by all of the tests that it has been possible to devise, the virus protein is homogeneous. I should hesitate to conclude as a result of any one test that a material is homogeneous and hence pure. However, when a material is found homogeneous by several quite different types of tests, I consider it highly significant. The various tests for homogeneity that have been applied to the virus protein are as valid as the cultural and microscopic tests used by the bacteriologist, hence it may be concluded that the essence of Koch's second postulate has been fulfilled. In addition, considerable

evidence of a more or less direct nature that the virus activity is a specific property of the protein has been obtained. In all of the work that has been done on tobacco mosaic virus protein in the writer's laboratory and that has been reported from other laboratories, not one bit of evidence that the purified virus protein is inhomogeneous or that the virus activity is due to material other than the high molecular weight protein has been obtained. If a decision be required at the present time, it is impossible from a chemist's standpoint or as a result of the application of Koch's postulates to conclude other than that the virus protein is actually tobacco mosaic virus.

However, just as the possibility exists that tuberculosis may some day be found to be due not to the tubercle bacillus but to something adsorbed on the organism, the possibility exists that the virus activity may be due not to the protein but to an impurity adsorbed on the protein that cannot be detected by means now at our disposal, but which may some day be detected by more refined methods. It is impossible to obtain final conclusive proof that any given material is pure, and the possibility that a certain property of any given material may be due to an impurity must always remain, regardless of the material. Insulin was discovered seventeen years ago, was crystallized twelve years ago, and has been subjected to extensive investigation. Although the biological activity of insulin is generally regarded as a property of the protein, there are workers who feel that the activity may be due to a dissociable group attached to the protein or to a separate entity adsorbed on the protein, and that eventually it may prove possible to separate the active agent from the protein. Should this ever prove possible or should it ever prove possible to separate the virus activity from the protein, I should regard it not as a catastrophe but as a most important and welcome advance. We would then be able to throw away 99.9 per cent of the virus protein or of the insulin protein and in the small remaining fraction we would retain all of the original activity. Now, it should be noted that by virtue of the experimental evidence that has already been accumulated with respect to these two proteins, the impurities or active agents could hardly be other than closely related proteins, hence it would still follow that the virus and insulin are proteins, but possessing activities a thousand or more times greater than the materials now known. According to present standards, such materials would be most amazing agents and their isolation would constitute a most important discovery. Although we

should recognize this possibility that the virus activity may eventually be found not a property of the high molecular weight protein, I think we should also recognize the fact that there is no reason to believe that such a situation actually prevails and that, according to all of the evidence available at present, we may conclude that the virus protein is actually the virus.

Isolation of the Virus Proteins of the Strains of Tobacco Mosaic Virus and of Other Viruses

Because of the life-like properties that are characteristic of viruses, the conclusion that the high molecular weight protein is the mosaic virus is fraught with implications of importance. Before discussing these, however, I should like to consider whether or not the general conclusion can be justified, firstly, with respect to the strains of tobacco mosaic virus and, secondly, with respect to different viruses. There is good evidence[48,49] that as ordinary tobacco mosaic virus multiplies within a host it occasionally mutates or in some manner becomes altered so that new and different strains of virus arise. These strains may be separated and isolated by means of an appropriate technique[48,50] and, although some occasionally revert to the ordinary strain, there is a definite tendency for them to remain as distinct strains of the mosaic virus. There are, therefore, several well recognized strains of tobacco mosaic virus. It was of considerable importance to determine whether or not plants diseased with strains of tobacco mosaic virus would contain high molecular weight proteins and, if so, whether or not these proteins would be similar to tobacco mosaic virus protein. The problem has been studied in the writer's laboratory and by Bawden and Pirie[38,51] in England. It was found that from plants diseased with strains of tobacco mosaic virus such as aucuba mosaic, enation mosaic, and the Holmes masked strain could be isolated high molecular weight virus proteins that were remarkably similar to tobacco mosaic virus protein but that differed in certain respects not only from each other but from the mosaic virus protein. For example, although the virus proteins of tobacco mosaic virus and its strains were found to have the same elementary chemical composition, optical rotation, crystalline appearance, and similar x-ray diffraction patterns and serological properties, it was possible to distinguish them by means of solubility and isoelectric point determinations, their reactions with clupein sulphate, and serologically by means of the

cross absorption technique.[23,38,51] The various strains of tobacco mosaic virus are characterized, therefore, by different although closely related high molecular weight proteins. Thus, when tobacco mosaic virus mutates or in some way becomes altered so that a new strain arises, this change is accompanied by the production of a new and slightly different virus protein. Inoculation of this new virus protein to susceptible hosts results not in the production of the ordinary disease and virus protein, but in the production of the new disease and of a virus protein of the same kind as that used as inoculum. This is exactly what would be expected to happen if the protein is the virus, and the fact that it actually happens serves as additional justification for the original conclusion that the protein is the virus.

Now let us examine the situation with respect to other viruses for, if tobacco mosaic virus is truly representative, it should be possible to isolate other viruses in the form of high molecular weight proteins. However, when the chemical method used for the isolation of the virus proteins of tobacco mosaic and its strains was first applied to plants affected by some of the less stable viruses such as those causing the tobacco ring spot, latent mosaic of potato, and severe etch diseases, it was not found possible to isolate high molecular weight proteins from such plants by this chemical method. These viruses are considerably less stable than tobacco mosaic virus and there was some indication that they existed in low concentration in the host. It seemed possible, therefore, that the chemical method might cause inactivation of these viruses due to their instability or that the method might not be sufficiently specific to separate a small amount of virus protein from a large excess of other proteins. The results demonstrated that the chemical method would have to be improved or a new method evolved, in order to work successfully with such viruses. Fortunately, about this time the development of the air-driven centrifuge reached a stage where it was possible to subject a hundred or more cc. of solution to high-speed centrifugation. In cooperation with Dr. Wyckoff,[52] it was found possible to isolate tobacco mosaic virus protein from the juice of diseased plants by means of differential high-speed centrifugation. The general method of differential centrifugation is not new, for it was used as early as 1922 by MacCallum and Oppenheimer[53] in work with vaccinia. It was used subsequently by Ledingham,[54] Craigie,[55] and Rivers[56] for the isolation and purification of the elementary bodies of vaccinia. Although high-

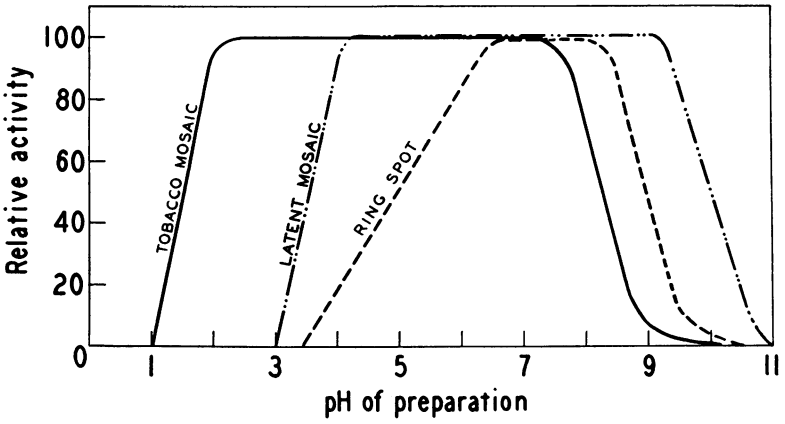

Fig. 7—pH stability range of activity of tobacco mosaic, tobacco ring spot, and latent mosaic of potato virus proteins. (Drawn from data of Best and Samuel,[32] Stanley[23,61] and Loring.[59])

speed centrifugation was used by Bauer and Pickels[57] to concentrate yellow fever virus, none of the smaller viruses had been isolated by the centrifugal method prior to 1936. Because of the ease and rapidity with which it was found possible to isolate tobacco mosaic virus protein by differential high-speed centrifugation, it seemed likely that the method should prove useful in the isolation of the less stable viruses.

In collaboration with Dr. Wyckoff,[58] batches of Turkish tobacco plants diseased respectively with tobacco ring spot, latent mosaic of potato, severe etch, and cucumber mosaic viruses were examined, and in every instance it was found possible to demonstrate the presence of a high molecular weight protein. Tobacco ring spot and latent mosaic virus proteins were isolated in sufficient quantity so that it was possible to study their physical, chemical, and serological properties. These properties were found to differ markedly not only from those of tobacco mosaic virus protein but also from each other. For example, ring spot virus protein causes the ring spot disease, appears homogeneous when examined in the ultracentrifuge, has a sedimentation constant of $S_{20}° = 115$, and is completely denatured and inactivated after standing for one hour at pH 3 or following a five minute exposure to a temperature of 64° C. This virus protein was found to be about 10,000 times more active than the infectious juice used as starting material. Dr. Loring[59] has found the latent mosaic virus protein to have its own definite and highly

characteristic set of properties. Bawden and Pirie[51] have isolated two stable strains of cucumber mosaic virus in the form of crystallizable high molecular weight proteins by chemical means. They have just recently isolated[60] from tomato plants diseased with bushy stunt another virus protein that differs from those previously described, in that it crystallizes in the form of dodecahedra. It should be emphasized that these proteins differ markedly in their physical, chemical, biological, and serological properties. For example, in Figure 7 is given the pH range of stability of three virus proteins. Tobacco mosaic virus protein is native and active between pH 2 and 8, latent mosaic virus protein between pH 4 and 9.3, and ring spot virus protein between pH 6 and 8. At more acid or alkaline reactions than those given for each of these virus proteins, they disintegrate, become denatured, and lose their virus activity.

Another point that should be emphasized is that the concentration reached by these several virus proteins in the same host differs markedly. In Figure 8 are given the amounts of virus protein that were isolated from 200 gm. of tissue diseased with different strains of tobacco mosaic virus and with other viruses.[61] It may be seen that in the same host, Turkish tobacco, the concentration level reached by tobacco mosaic virus protein is greater than that of its two strains, aucuba mosaic and the masked strain, is 268 times greater than that of tobacco ring spot virus protein, and is 2000 times greater than that of cucumber mosaic virus protein. It may be noted that in the case of the tobacco mosaic disease in Turkish tobacco plants as much as 80 per cent of the total protein of the plant has been isolated in the form of the virus protein. Another fact of importance is that the same virus protein reaches a different level of concentration in different hosts. Thus, the level reached by tobacco mosaic virus protein in Turkish tobacco is 400 mg., in tomato 260 mg., and in spinach 30 mg. per 200 gm. of plant material. There is also included in this figure the amount of the homogeneous heavy protein carrying virus activity that Beard and Wyckoff[62] were able to isolate by means of differential high-speed centrifugation from 200 gm. lots of the warty tissue of rabbits diseased with the Shope papilloma virus. The concentration of this material is of the same order as that of some of the less abundant plant virus proteins. This material, that of the Rous sarcoma virus isolated by Claude,[63] the material containing a nucleoprotein isolated by Janssen[64] from tissue infected with the foot-and-mouth disease virus and the elementary bodies of vaccinia are now

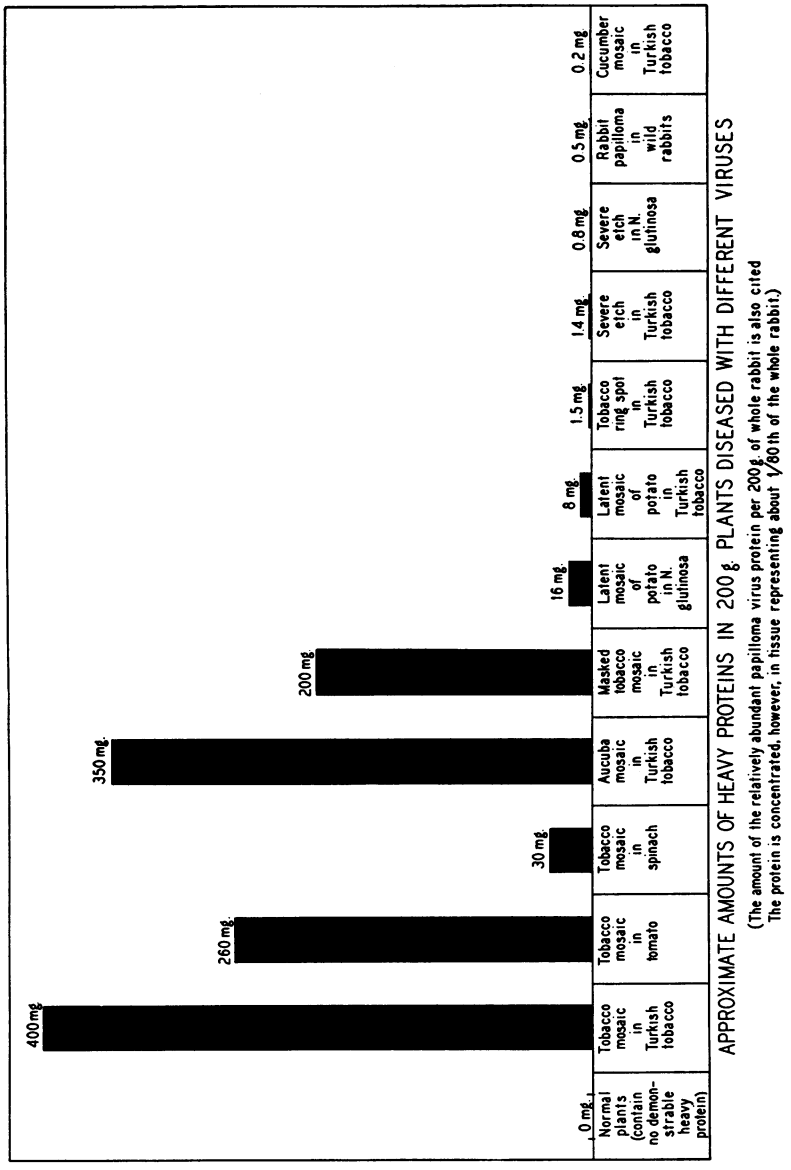

Fig. 8—Approximate amounts of heavy proteins in 200 gm. of tissue diseased with different viruses.

obtainable in reasonably large amounts. It seems likely that it will not be long before it will be possible to make a decision as to whether or not these materials are similar to the plant virus proteins. We may conclude from the amounts of the different plant virus proteins that have been isolated that a given virus may reach different concentration levels in different hosts and that in the same host different concentration levels are reached by different strains of the same virus and by different viruses. The amount of virus protein produced in a host is therefore dependent not only upon the virus but also upon the host.

Recovery of Tobacco Ring Spot Diseased Plants

In this connection I think that I should mention the interesting recovery phenomenon exhibited by Turkish tobacco plants diseased with tobacco ring spot virus. Following the initial violent attack, these plants appear to recover and present a normal appearance. This phenomenon was described by Price,[65] who showed that such recovered, apparently normal plants still retained virus, although in a reduced amount, and that they were immune and could not be reinfected. These recovered plants have now been examined and from such plants there has been isolated a virus protein that appears to be identical in all respects with that isolated from badly diseased plants. As was expected in view of Price's work, the amount of the protein in recovered plants was found to be only about one-sixth that in badly diseased plants. Recovery, therefore, appears to consist of some mechanism by means of which the amount of virus is reduced in concentration to a level that no longer causes disease symptoms. I have mentioned this phenomenon because it is a striking demonstration of the persistence of virus in recovered hosts, because it shows the effect of different levels of virus concentration in the same host, and because additional evidence correlating protein with virus was secured by the isolation of the same virus protein from apparently normal although immune plants.

Discussion

The isolation of several virus proteins from tissues diseased with different viruses and the demonstration that these virus proteins possess highly characteristic physical, chemical, biological, and serological properties that differ not only from each other but also from those of tobacco mosaic virus protein serve as additional justification for relating virus

activity to protein. We see, therefore, that all of the information that is now available regarding the homogeneity of the virus proteins, the relationship of virus activity to protein, the isolation of different strains of the same virus in the form of different although closely related proteins, and the isolation of different viruses in the form of quite different and highly characteristic proteins, indicates that the virus proteins are in fact the viruses themselves and that if a decision be required at present the only conclusion that is possible, based on experimental evidence, is that these proteins are the viruses.

Nevertheless, some workers refuse to entertain the idea that the protein may be the virus, because they dislike to consider that a protein molecule may possess certain properties such as the ability to reproduce and to mutate, properties that they like to consider as characteristic of living things. However, it is foolish to disregard experimental facts and to attempt to come to a decision solely on the basis as to whether a given substance does or does not possess properties characteristic of living things, for there is not a single property that has been considered characteristic of living things that may not be duplicated in a recognized non-living system. I do not mean by this that living things do not differ from non-living things. I do mean, however, that I feel we should not be too strongly influenced by the conventional criteria of life. As we go from the admittedly non-living to the admittedly living, I think that there must be a transition stage where there are entities that may possess some properties that are considered characteristic of living things and some properties that are considered characteristic of non-living things. What could fill this place more simply and logically than the high molecular weight virus proteins that are intermediate in complexity between the protein enzymes and hormones, the wonderful properties of which we already recognize, and the system of proteins that we call protoplasm and that constitutes life. There is evidence that even within the virus group there is a gradual increase in complexity of structure from the small nucleoproteins to the more elaborate elementary-body type of virus. There is, however, no sharp break despite the fact that in certain respects the structure of the latter may resemble that of a cell-type organism as much as it resembles that of the smaller viruses. I consider it unimportant whether we call the virus proteins molecules or organisms and this evening I have referred to them as molecules solely because of the accident of my training as a chemist. However, I consider

the correct recognition of their fundamental properties a matter of extreme importance.

I should like to point out that, although the endowment of a protein molecule with virus properties marks a new and previously unrecognized property of proteins, it cannot, in view of the unusual properties of the protein enzymes and protein hormones that have been recognized in recent years, be regarded as a totally unexpected property. Furthermore, although the recognition may constitute an advance, it certainly is not particularly enlightening, for in placing the secret of viruses within a protein molecule Nature has selected the least understood and the most mysterious of all the compounds with which the chemist works, for the structure of not one protein is known. In recent years the protein molecule has attracted the interest of workers in different fields and this community of interest is especially noteworthy at the present time in the case of the virus proteins. They interest the pathologist since they cause disease, and the bacteriologist because of their small size and because they possess certain properties that have been regarded as belonging to organisms. The chemist is attracted to them because, although they have many of the properties of molecules, they possess in addition properties that have not hitherto been ascribed to molecules. The physicist is interested in them because of their properties as macromolecules and because some virus proteins show that interesting layering phenomenon that has been called a new property of matter. They are of interest to the biologist because they possess properties that have been regarded as characteristic of both living and non-living things. The geneticist is interested in them because they undergo a phenomenon similar to mutation and thus may possibly permit a study of the nature of mutation from a new viewpoint. Lastly, they interest the philosopher because they permit him to enter with renewed vigor upon a discussion of that age-old question of "What is life?" The virus proteins thus bid fair to become the common meeting ground of scientists. The advance so far has been merely a more exact definition of the problem that confronts us, the problem of the protein molecule. The most interesting and important advances and at least one fundamental discovery lie ahead and depend upon the ability of workers in different fields of science to explore successfully the protein molecule.

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